RESEARCH PAPERS

EFFECT OF ISOLATION AND CRYSTALLIZATION ON THE ELECTROPHORETIC MOBILITY OF β-LACTOGLOBULIN AS DETERMINED BY PAPER ELECTROPHORESIS

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SUMMARY

To study the electrophoretic properties of milk proteins under conditions as nearly similar as possible to those found in milk, a paper strip electrophoresis apparatus was designed which required small volumes of electrolyte and in which electro-osmosis could be substantially reduced. Using this apparatus, it was shown that β-lactoglobulin could be isolated from milk, crystallized, and returned to its native environment without grossly affecting its initial charge density.

Inherent in all protein isolation procedures is the supposition that the end product obtained has an exact counterpart in the native state of the system from which it was separated (14). With the increasing refinement of analytical techniques, many pure crystalline proteins have been shown to be heterogeneous in respect to either their chemical or their physical properties, or both (7, 10, 11, 13). One possible source of the observed micro-heterogeneity in crystalline protein preparations has been ascribed to the effects of the purification techniques employed in their isolation (4, 12). These facts tend to cast doubt upon the initial surmise that the crystalline proteins have exact counterparts in the system from which they were isolated. Comparison of the physical and chemical properties of proteins before and after isolation is difficult, or impossible, in most cases. However, some work has been done in this field.

Studies of the oxygen dissociation curves (2) and the spectral adsorption (1) of hemoglobin in the red blood cell and in systems containing isolated hemoglobin have indicated that this protein in its native environment has properties different from those observed after isolation has been effected.

In the course of a study of the protein interactions that occur in milk during the processing and storage of milk products, methods were developed for investigating the electrophoretic mobilities of the milk proteins in milk serum. A comparison of the electrophoretic mobilities of a sample of crystalline β-lactoglobulin and the native whey proteins of milk was made in protein-free whey. This paper reports results obtained during this study, which indicate that an isolation and crystallization technique now commonly employed to produce pure β-lactoglobulin does not affect the charge distribution on the surface of the molecules of this protein. Crystalline β-lactoglobulin is shown to have an electrophoretically similar counterpart in the native state of the system from which it was isolated.

Since the electrophoretic apparatus used in this study is somewhat different from types now in common use, its design and operation characteristics are described in some detail.

EQUIPMENT AND MATERIALS

A. Paper strip electrophoretic apparatus. The apparatus used in this investigation is a variation of the simple design of Kunkel and Tiselius (9), in which an electrical potential is applied through large open electrode vessels to the ends of a buffer-dampened paper strip sandwiched between two pieces of plate glass. This design was modified to eliminate the bulky electrode compartments and minimize the amount of electrolyte used for an analysis. The electrical potential was applied to the paper strip through reversible silver–silver chloride electrodes embedded in blocks of agar containing KCl.

The apparatus was constructed from two blocks of Lucite,[1] each 24 by 4 in. One block was ¾-in. thick, the other ½-in. thick. Two rectangular electrode compartments 4⅛ by 2⅛ in. were milled out of the ends of the thickest block. The distance between the inside edges of the compartments was 17⅜ in. Electrodes consisting of four ⅜-in. o.d. coils of 1/16-in. silver wire were placed in each compartment and connected through a 1/16-in. hole drilled through the side of each well to terminals mounted on the side of the block. After the electrodes were electrolytically plated with silver chloride, the wells were filled to the top with a hot 2% agar solution containing 1% KCl. On cooling, the agar solidified around the silver–silver chloride coils to form a stable reversible electrode system.

To maintain the apparatus at constant temperature during operation, a water jacket 23 by 5 by ¼-in. was cemented onto the outside face of each block.

The apparatus was held together by six 2-in. C clamps.

Direct current was obtained from a Spinco Duostat [1] power pack.

Figure 1 is a photograph of the apparatus as used in the described experiments.

B. Protein-free whey. A solution having very nearly the some composition and ionic activity as the phase in which the milk proteins are found in their native state, was prepared by dialyzing 200 ml. of distilled water against 40 liters of skimmilk. The dialysis was carried on for five days with intermittent agitation at 5°C. Ten milliliters of toluene were added to the milk before dialysis, to prevent bacterial growth.

C. Native whey protein concentrate. Skimmilk was centrifuged in the A Rotor of a Model E Spinco [1] Ultracentrifuge for 60 min. at 57,000 × G and 23°C., to remove colloidal caseinates. The supernatant solution was further centrifuged in the same rotor for an additional 6 hr. at 150,000 × G and 23°C., to concentrate the whey proteins. After centrifugation, all material except the pellet and bottom 1 ml. of solution was removed from the tubes. The pellets were broken up into the residual solution in the tubes and allowed to stand at room temperature for approximately ½ hr. The material was collected and centrifuged in a clinical centrifuge for 15 min. The clear supernatant solution

[1] The mention of trade names in this paper is for identification and does not imply endorsement of the product or its manufacturer by the U. S. Department of Agriculture.

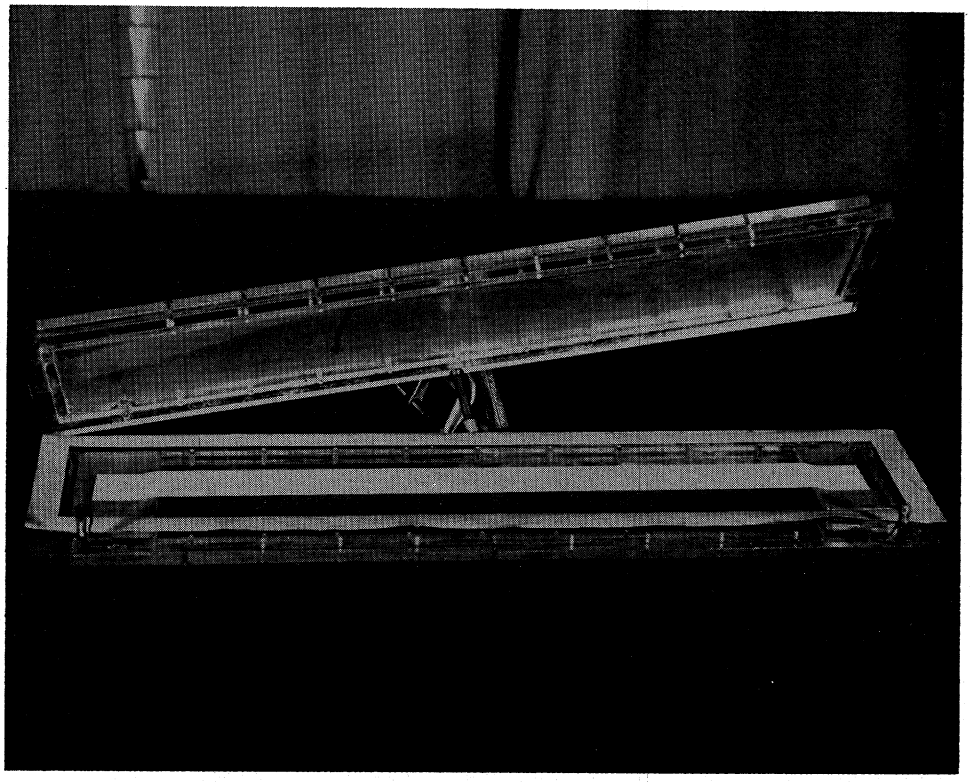

FIG. 1. Photograph of paper electrophoresis apparatus equipped with reversible silver–silver chloride electrodes imbedded in agar gel.

representing concentrated whey proteins in unmodified whey was held under refrigeration in an atmosphere of toluene for electrophoretic analysis. These solutions were found to be relatively unstable, and were not stored for periods in excess of three days.

D. Purified β-lactoglobulin. Crystalline β-lactoglobulin was prepared according to the method of Gordon *et al.* (6). After triple recrystallization the crystals were collected by centrifugation and lyophilized. The dried material was stored under refrigeration. Subsequent analysis showed this preparation to be electrophoretically homogeneous in phosphate buffer at pH 7.5.

EXPERIMENTAL METHODS

The electrophoretic characteristics of the proteins used in this study were determined on strips of Whatman No. 1 filter paper 22 in. long and ranging from ½ to 3 in. in width. The type of strip selected was wet with the solution in which the analysis was to be carried out. Approximately 2.5 ml. of solution were required for each inch of strip width. Excess fluid in the strip was removed by blotting between sheets of filter paper. The damp strip was then placed on the surface of the electrode-containing plastic block and the ends of the strip

were brought into contact with the agar surfaces of the electrodes. Electrical contact between the agar and the paper strip was established by adding an additional drop of electrolyte to each end of the strip. A sample of the protein solution to be analyzed was placed on the paper near the center of the strip in the form of a spot or a line, using either a micropipette or a Spinco [1] applicator. Sample size used ranged from one lambda to ten lambda, depending on the protein concentration in the sample analyzed.

Evaporation of water from the strip during electrophoresis was controlled by placing a Teflon [1] gasket ¼-in. wide and approximately 0.01 mm. thick around the outer edge of the block. The strip was then covered with the second Lucite [1] block and two blocks were clamped together, using six 2-in. C clamps. Uniform pressure on the strip is essential for proper operation.

The apparatus was equilibrated by allowing water of the desired temperature to circulate through the jackets for a 15-min. period before starting the analysis. Water temperatures ranging from 10 to 23°C. were used during the study. Direct current was then passed through the strip long enough to achieve sufficient protein migration on the strip, the intervals ranging from 1 to 6 hr. On completion of the runs the strips were removed from the apparatus, dried in a circulating air oven for 20 min. at 130°C., and stained with bromphenol blue (9).

The electrodes were reversed in polarity after each run, to maintain their silver chloride coating. If the buffers used in the analysis did not contain chloride, the electrodes were regenerated electrolytically after each week of operation.

The operational efficiency of the apparatus was determined by comparing strips obtained by its use with those obtained using a commercial model of the Durrum type apparatus (5). The fact that the new apparatus was water-cooled enabled much higher potentials—up to 720 volts—to be placed across the strip. Therefore, equivalent migration and resolution could be obtained in much shorter time than required for the Durrum type apparatus.

Restriction of electro-osmotic flow of water in the strip was studied by operation of the apparatus in positions other than horizontal.

The mobility of the crystalline β-lactoglobulin dissolved back into whey was compared with the mobility of the whey proteins not removed from their native environment. Whatman No. 1 paper strips were wet with protein-free whey, and samples of crystalline β-lactoglobulin dissolved in whey and native whey protein concentrates were allowed to migrate through the strips under the influence of identical electrical potentials. The rate of migration of the two materials in the native environment of the milk proteins was compared.

To determine the effect of the presence of colloidal caseinates on β-lactoglobulin in its native environment, small amounts of the caseinates initially centrifuged out of the milk were added back to the native whey-protein concentrate before electrophoretic analysis in protein-free whey.

RESULTS

During preliminary studies it was found that whey protein mixtures could be resolved and the electrophoretic mobilities of proteins validly compared by

use of paper electrophoresis apparatus equipped with reversible electrodes imbedded in a gelled electrolyte.

With an apparatus of this type, the electro-osmotic flow of water in the paper strips (3, 8) could be greatly reduced and, in most cases, completely eliminated. This was accomplished by operating the apparatus in a vertical position, allowing the force of gravity to act against the electro-osmotic forces operating in the paper strip. Using lactose as a neutral marker compound, the extent of electro-osmotic flow in the strip was determined. If the strips were wet with veronal buffer pH 8.6, ionic strength 0.10, the water moved 0.6 cm. per hour towards the anode, with a potential of 700 volts impressed across the strip oriented in a horizontal plane. Under the same conditions, with the strip oriented in a vertical position with the cathode up, the rate of marker migration was 1.3 cm. per hour. With the strip in a vertical position, with the cathode at the lower end of the strip, no electro-osmotic effects were observed.

The low electrolyte requirement of the apparatus also made possible the determination of the mobility of some of the whey proteins in protein-free whey. Crystalline β-lactoglobulin dissolved in protein-free whey was found to migrate through whey exactly as fast as the fastest component in the native whey protein concentrate, when both were subjected to fields of equal electrical potential, as illustrated in Figure 2.

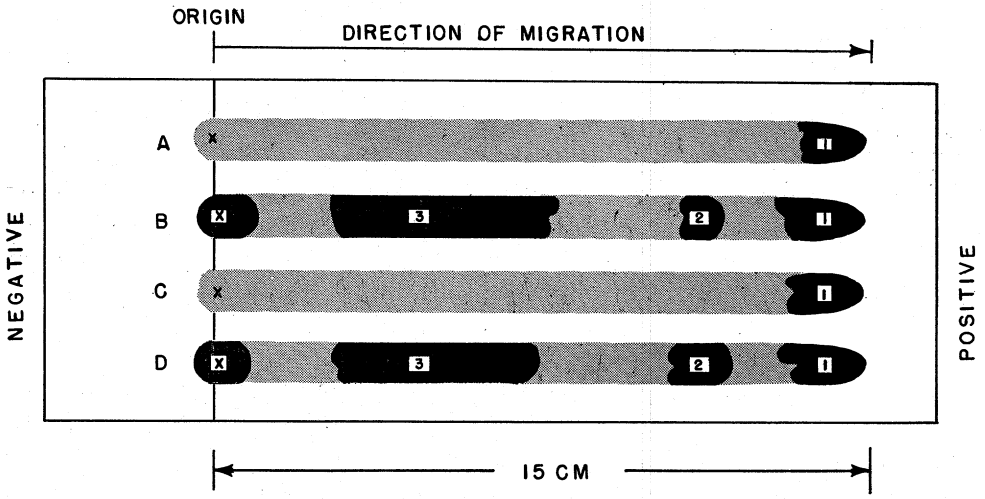

FIG. 2. Electrophoretic migration of milk proteins on a paper strip saturated with protein-free whey. 5λ of 4% solution of crystallized β-lactoglobulin in protein-free whey were spotted at x on A and C and 5λ of a native whey protein concentrate of approximately 8% protein were spotted at x on B and D. Diagram indicates results after 6 hr. of electrophoresis at 20°C., with a potential of 700 volts across ends of strips. Spots numbered one are β-lactoglobulin. Spots 2 and 3 are unidentified whey proteins. Shaded areas represent trails of proteins adsorbed on strip.

Adding the caseinate complex back to the native whey protein concentrate caused a slight shift in the distribution of the fastest component towards the point of origin, as can be seen in Figure 3. The leading edge of the zone con-

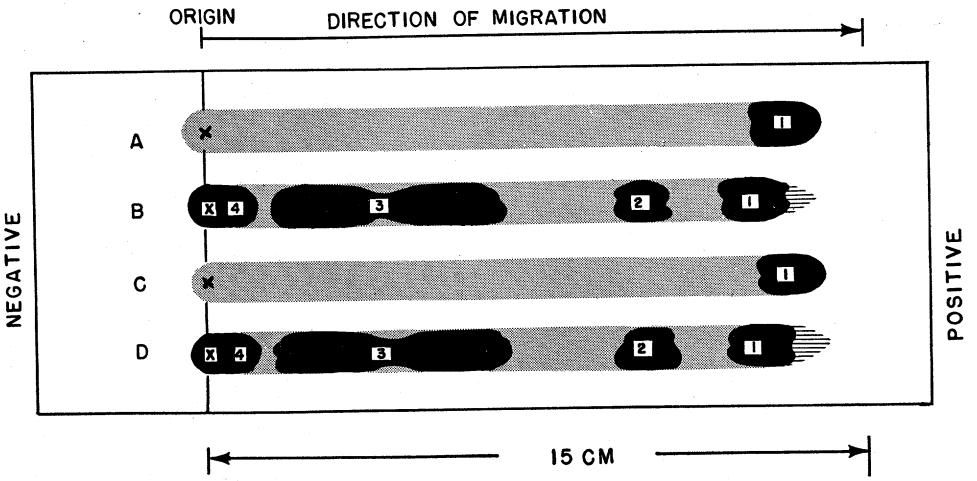

FIG. 3. Electrophoretic migration of milk proteins on a paper strip saturated with protein-free whey. 5λ of a 4% solution of crystallized β-lactoglobulin in protein-free whey were spotted at x on A and C and 5λ of a native whey protein concentrate of approximately 8% protein plus 5λ of a 4% solution of casein in whey were spotted at x on B and D. Diagram indicates results after 6 hr. of electrophoresis at 20°C., with a potential of 700 volts across ends of strips. Spots numbered one are β-lactoglobulin. Spots 2 and 3 are unidentified whey proteins. Spots 4 are unidentified whey proteins plus casein. Shaded areas represent trails of proteins adsorbed on strip. Parallel lines indicate zones of low protein concentration.

taining the fastest component maintained the rate of migration noted before the addition of the caseinate. The amount of fast-moving protein shifted toward the origin depended on the diameter of the caseinate spot which remained at the point of application during the analysis. This possibly indicates that the β-lactoglobulin might have been retarded by a reversible protein-protein interaction with the caseinate as it migrated across the spot of application at the start of analysis.

DISCUSSION

Observations made with the equipment described in this report lend some further support to the supposition that β-lactoglobulin isolated from milk in a crystalline condition has undergone no radical change in physical properties. Although electrophoretic mobilities offer only one criterion for comparison, it is noteworthy that the electric charge density of the isolated protein is unchanged after return to its native environment.

Evidence of interaction between β-lactoglobulin and the casein micelles observed on the whey-saturated strips does not mean this situation exists in milk. The caseins are bound to the paper strip with forces sufficiently high to possibly change their physical structure to the point where interaction with β-lactoglobulin will occur.

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